19. VITAMIN B_1 AND BACTERIAL OXIDATIONS

1. DEPENDENCE OF ACETIC ACID OXIDATION ON VITAMIN B_1

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Vitamin B_1 is known to be a necessary factor for the growth of certain bacteria [Knight, 1937, 1, 2; cf. also Tatum *et al.* 1936; Barron & Lyman, 1939; Nilsson *et al.* 1938]. Such bacteria can be grown on media containing extremely minute quantities of the vitamin, and may even be trained to grow in the absence of the vitamin [Wood *et al.* 1938], in which case the organisms apparently may acquire the ability to synthesize it [Silverman & Werkman, 1939, 2]. The metabolism of bacteria grown on vitamin B_1-deficient media may be greatly enhanced by the addition of small quantities of the vitamin to suspensions of the organism. This has been shown by Hills [1938] in the case of *Staphylococcus aureus*, and by Silverman & Werkman [1938] in the case of propionic acid bacteria. The former author reported a marked stimulation of the oxidation of pyruvate and of the anaerobic breakdown of pyruvate by *Staphylococcus* on the addition of the vitamin. Silverman & Werkman [1938; 1939, 3] found that the rate of anaerobic breakdown of pyruvic acid by propionic acid bacteria and by *Lactobacillus mannitopoeus* is increased on the addition of the vitamin to washed suspensions of the organisms.

We have carried out a systematic study of the effects of vitamin B_1 on the oxidative powers of propionic acid bacteria grown on vitamin B_1-deficient media and of the various factors which influence the action of the vitamin. It is proposed to deal in this paper with the effects of the vitamin on various oxidations stimulated by these bacteria. We have already reported that the vitamin appears to be essential for the oxidation of acetic acid by these organisms [Quastel & Webley, 1939].

Preparation of vitamin B_1-deficient propionic acid bacteria

We have used a strain of propionic acid bacteria obtained from the National Collection of Type Cultures (Ref. No. 4759). The organism is grown on Petri dishes containing a medium deficient in vitamin B_1. This medium is prepared as follows. A mixture of 20 g. 'Difco' peptone, 6 g. NaCl and 40 g. agar-agar is dissolved in 1 l. of distilled water, the mixture being warmed until solution has taken place. *N* NaOH solution is now added until the *p*H is 9·0, using bromothymol blue as indicator, and the mixture is autoclaved at 125° for 1 hr. The medium is finally filtered through absorbent cotton wool and the *p*H adjusted to 6·6. This medium may be autoclaved many times without its consistency, after cooling in Petri dishes, being appreciably affected. Occasionally we have found it necessary to autoclave the medium a second time at *p*H 9·0 to reduce the vitamin B_1 content to the low levels required for the preparation of the vitamin-deficient bacteria.

The plates are spread with an 18 hr. culture of the propionic acid bacteria which have been previously grown on the vitamin-deficient medium. The organism is grown aerobically at 37° for 18 hr., after which it is washed off the plates with sterile 0·16 *M* saline. The suspension of organisms is washed twice with saline by centrifuging. It is finally suspended in saline and stored at 0°. 1 ml. of the suspension is usually made to contain about 10 mg. dry weight of the bacteria.

Measurement of the respiration of the bacteria

This is accomplished with the use of the Barcroft, and Warburg, respiratory apparatus. 1 ml. of the bacterial suspension is usually employed and to this are added solutions of phosphate buffer, substrate etc., to produce a final volume of 3 ml. The measurements of O_2 uptake are carried out at 37° at *p*H 7·4 in an atmosphere of air. The experiments are generally run for 1 hr.

Measurement of the weight of bacteria

This is carried out by means of the Spekker photoelectric absorptiometer. The opalescence of a known weight of the bacteria suspended in saline is measured on the instrument, using a standard light filter, and a calibration curve is drawn relating opalescence to bacterial weight. The curve is linear for densities of the organism not exceeding 0·25 mg. dry weight per ml. With the aid of this curve it is a simple matter at the termination of each experiment to dilute the contents of the respiration flask until a suitable density of the organism is obtained, to determine the opalescence on the absorptiometer and to estimate from the curve the weight of bacteria present in the suspension.

The effect of vitamin B_1 on the oxidation of acetate by vitamin B_1-deficient propionic acid bacteria

Acetic acid is known from the work of Stone *et al.* [1936] to be a hydrogen donator in presence of propionic acid bacteria. Little is known of the metabolism of acetic acid in presence of these bacteria, but Wood *et al.* [1937] conclude that their evidence, derived from analyses of the products of glucose fermentation, points to the conversion of acetic acid into succinic acid.

The addition of acetate to suspensions of vitamin B_1-deficient propionic acid bacteria increases the rate of O_2 uptake. The further addition of vitamin B_1 results in a large acceleration of the rate of oxidation of acetate. A typical result is shown in Table 1. The rate of O_2 uptake by the bacteria in the presence of acetate and vitamin B_1 is linear. As the oxidation proceeds, the *p*H of the solution in the respiration flask steadily increases, sodium bicarbonate and carbonate being formed. The vitamin has but little effect on the respiration of the bacteria in the absence of added substrates.

Table 1

Each manometer vessel contained 1·0 ml. suspension of freshly washed and freshly grown *B. acidi propioni*, 0·5 ml. *M*/5 sodium phosphate buffer *p*H 7·4, the amounts of substrates and vitamin B_1 stated below and 0·16 *M* saline to bring the volume to 3 ml. O_2 uptakes were determined in an atmosphere of air at 37° for a period of 2 hr.

Substrate added	Vitamin B_1 added μg.	O_2 uptake in 2 hr. μl.
Nil	Nil	197
0·3 ml. *M*/10 Na acetate	Nil	563
Nil	0·25	245
0·3 ml. *M*/10 Na acetate	0·25	1202

Variation of the velocity of O_2 uptake by propionic acid bacteria in presence of acetate with the concentration of vitamin B_1

The velocity of respiration of the vitamin B_1-deficient bacteria in presence of 0·01 *M* sodium acetate rises sharply as the concentration of vitamin B_1 present is increased. A maximum is reached at a concentration of approximately 30 μg. vitamin B_1 per 100 ml., i.e. 7×10^{-7} *M*. The velocity of O_2 uptake in presence of acetate and in absence of added vitamin B_1 is variable, depending on the conditions of culture and age of the organism. Usually the addition of acetate to a suspension of vitamin B_1-deficient bacteria increases the rate of O_2 uptake, the increase being most probably determined by the small quantities of the vitamin still present in the organism. The variation of the rate of O_2 uptake of the vitamin-deficient bacteria in presence of 0·01 *M* sodium acetate with variation of the amounts of vitamin B_1 added to the suspension of cells is shown in Fig. 1. It

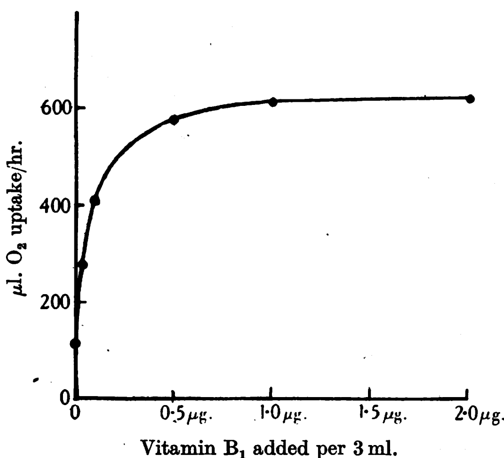


Fig. 1. O_2 uptake by propionic acid bacteria in presence of 0·01 *M* Na acetate and varying quantities of vitamin B_1.

will be noted that a large increase in the rate of respiration in the presence of acetate is secured by the addition of only 0·1 μg. vitamin B_1 to the 3 ml. of cell suspension in the respiration flask.

If propionic acid bacteria are grown on a peptone-agar medium to which vitamin B_1 has been added, suspensions of these bacteria oxidize acetate at a much greater rate than holds when the bacteria are grown on vitamin B_1-free medium. Moreover, the addition of the vitamin to suspensions of bacteria grown on a vitamin B_1-rich medium does not increase the rate of oxidation of acetate.

O_2 uptake and bacterial count

The increased O_2 uptake secured by the addition of vitamin B_1 to suspensions of vitamin B_1-deficient bacteria in presence of acetate is not due to multiplication of the organisms in the respiration flask under our experimental conditions. This is shown by adding the same number of cells to a phosphate-acetate medium, in the absence and in the presence of vitamin B_1, measuring the O_2 uptakes at 37° for 1 hr. and making bacterial counts at the end of this period using a blood-counting chamber. A representative result is shown in Table 2.

Table 2

Conditions as in Table 1

Substance added to 1 ml. bacterial suspension	µl. O_2 consumed	No. of bacterial cells present at end of experiment
Nil	120·9	$10·6 \times 10^9$
0·25 µg. vitamin B_1	152·6	$9·4 \times 10^9$
0·01 *M* Na acetate	380·0	$9·8 \times 10^9$
0·01 *M* Na acetate + 0·25 µg. vitamin B_1	821·3	$9·5 \times 10^9$

Oxidation of acetic acid by propionic acid bacteria

Analyses were made of the acetic acid remaining in the respiration flask after the consumption of O_2 by the bacterial suspension, and the amount of acetic acid which had disappeared was compared with the amount of O_2 taken up.

Acetic acid was estimated in the following manner. A mixture of 3 g. anhydrous Na_2SO_4 and 1·5 ml. H_2SO_4 (2 parts conc. H_2SO_4 to 1 of water) was placed in a small pyrex flask and to this were added 2 ml. of the fluid in the respiration flask after the bacteria had been removed by centrifuging. The volume was made up to 6 ml. with distilled water and the flask was connected to a worm condenser and then to a Liebig condenser, ground glass joints being used throughout. The worm condenser and its outlet to the Liebig condenser were swathed with cotton wool. The end of the Liebig condenser dipped into 30 ml. of distilled water contained in a receiving flask. Distillation was carried out for 20 min. using a microburner under the distillation flask. The worm condenser prevented droplets of the fluid in the distillation flask from passing into the receiving vessel, but condensation of the steam containing the acetic acid took place only in the Liebig condenser. The microburner was removed after 20 min. distillation, the apparatus was disconnected, 5 ml. water were added to the distillation flask via the worm condenser and the apparatus was reconnected. Distillation was carried out for a further 20 min., after which the microburner was finally removed, and 30 ml. water were poured down the Liebig condenser (for washing purposes) into the receiving flask. The volume of fluid in this flask was finally 70 ml. The acetic acid present was titrated with CO_2-free N/100 NaOH, using a constant quantity of bromothymol blue as indicator. The method gave accurate and consistent results as long as the volumes mentioned above were adhered to.

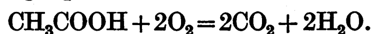
Table 3

Substance added to the bacterial suspension	µl. O_2 consumed in 2 hr.	µl. O_2 consumed due to the acetate	µl. acetic acid utilized	Ratio $\frac{O_2 \text{ consumed}}{\text{Acetic acid utilized}}$
Exp. 1				
Nil	197	—	—	—
0·25 µg. vitamin B_1	245	—	—	—
0·01 *M* acetate	563	366	193	1·90
0·01 *M* acetate + 0·25 µg. vitamin B_1	1202	957	486	1·97
Exp. 2				
Nil	195	—	—	—
20 µg. cocarboxylase	221	—	—	—
0·01 *M* acetate	446	251	132	1·90
0·01 *M* acetate + 20 µg. cocarboxylase	1397	1176	591	1·99

Typical results giving the ratio of O_2 consumed by the bacteria to the acetic acid utilized are given in Table 3. The conditions were those quoted in Table 1. The acetic acid utilized by the bacteria is expressed in terms of gas (i.e. 1 g. mol. ≡ 22·4 l. gas).

The results show that the ratio of O_2 consumed to acetic acid utilized, whether vitamin B_1 has been added to the suspension of vitamin-deficient propionic acid bacteria or not, is approximately 2·0.

This value is to be expected if complete oxidation of acetic acid occurs according to the following equation:



$$CH_3COOH + 2O_2 = 2CO_2 + 2H_2O.$$

Effects of vitamin B_1 on the oxidation of fatty acids by vitamin B_1-deficient propionic acid bacteria

The lower fatty acids, formic, propionic and butyric, are oxidized by propionic acid bacteria, but the addition of vitamin B_1 has a much less marked effect on the oxidation of these acids than it has upon that of acetic acid. Representative results are shown in Table 4. It will be seen that whereas the rates of respiration

Table 4

O_2 uptakes of vitamin B_1-deficient propionic acid bacteria in the presence of the Na salts of fatty acids and in the presence and absence of vitamin B_1. Each vessel contained 1 ml. freshly washed suspension of the bacteria, 0·5 ml. *M*/5 Na phosphate buffer *p*H 7·4, 0·1 ml. of a solution containing 0·01 *M* $MgCl_2$ and 0·024 *M* KCl, 0·3 ml. *M*/10 solution of the Na salt of the fatty acid, 0·2 ml. of the solution of vitamin B_1 and 0·9 ml. saline. Respiration measured in air at 37°. Q_{O_2} = μl. O_2 uptake per hour per mg. dry wt. of bacteria.

	Q_{O_2}	
Substrate	In absence of added vitamin B_1	In presence of 4 μg. added vitamin B_1
Nil	10·5	12·4
0·01 *M* Na formate	22·5	24·9
0·01 *M* Na acetate	23·4	65·3
0·01 *M* Na propionate	20·7	37·8
0·001 *M* Na butyrate	19·0	25·1

in the presence of the fatty acids and in absence of added vitamin B_1 are approximately the same, the rates differ most markedly in the presence of the vitamin. The addition of the vitamin has but little effect on the oxidation of formate and butyrate, but is definite with propionate and most marked with acetate. The conditions of the experiments, typical results of which are shown in Table 4, are different from those mentioned earlier in that a mixture of magnesium ions and potassium ions was added to the solution in the respiration flask. The reasons for the addition of these ions will be given in a later paper.

It is of interest to note that Stone *et al.* [1936] have shown that resting propionic acid bacteria activate acetic and propionic acids to reduce methylene blue under anaerobic conditions, whilst relatively little or no activation takes place with formic and butyric acids.

Is acetate oxidized via pyruvate in presence of propionic acid bacteria?

It follows from the results quoted in Table 4 that not all oxidations brought about by vitamin B_1-deficient propionic acid bacteria are materially affected by the addition of vitamin B_1. This brings up the question as to whether the accelerating action of vitamin B_1 is primarily due to its effect upon the oxidation

of an intermediate substance common to the breakdown products of acetic and propionic acids but not to those of formic and butyric acids. The obvious possible intermediate is pyruvic acid, the rate of oxidation of which by vitamin B_1-deficient propionic acid bacteria was found to be much increased by the addition of vitamin B_1, as is seen in Table 5.

Table 5

Conditions as in Table 4

Substrates added to bacterial suspension	Q_{O_2}
Nil	12·2
4 μg. vitamin B_1	14·5
0·01 *M* Na pyruvate	24·4
0·01 *M* Na pyruvate + 4 μg. vitamin B_1	42·8

The fact that propionic acid bacteria readily convert lactic acid into propionic acid makes it likely that the reverse process can take place, as pointed out by Stone *et al.* [1936]. It may be shown, with the use of vitamin B_1-deficient bacteria, that oxidation of propionic acid leads to the accumulation of a ketonic acid. When vitamin B_1 is added to the system the amount of ketonic acid accumulating is much diminished. Although the ketonic acid has not been isolated, the fact that it disappears in the presence of vitamin B_1 makes it most probable that the ketonic acid in question is pyruvic acid. This is shown much more clearly when glucose is used as the substrate for oxidation in presence of the vitamin-deficient bacteria. Here there is a rapid formation of a ketonic acid, the accumulation of which is greatly diminished by the presence of vitamin B_1. Its identity with pyruvic acid is made reasonably certain by the fact that Wood *et al.* [1937] have isolated this ketonic acid from the products of breakdown of glucose by the propionic acid bacteria. No other aldehydes or ketones were detected by these authors. The same phenomenon may be shown when lactate or glycerol is used as substrate for oxidation with the vitamin-deficient bacteria.

It is of some interest that the yield of pyruvic acid from lactic acid, when this is oxidized by the propionic acid bacteria, is much less than that from glucose. This shows that the oxidation of glucose does not depend on preliminary fission into lactic acid, and lends support to the view expressed by Wood *et al.* [1937] that the metabolism of glucose by the propionic acid bacteria follows the paths which are followed in muscle and yeast.

Representative results showing the rates of accumulation of pyruvic acid in the absence and presence of vitamin B_1 are given in Table 6. The yield of pyruvic

Table 6

Conditions as in Table 1

Substrates added to bacterial suspension	Vitamin B_1 added μg.	Dry wt. of bacteria mg.	Pyruvic acid liberated in 1 hr. μl.
Nil	Nil	10·5	Nil
Nil	4	10·5	Nil
0·01 *M* glucose	Nil	11·7	139·8
0·01 *M* glucose	4	11·7	86·0
0·01 *M* Na lactate	Nil	7·5	40·3
0·01 *M* Na lactate	4	7·5	6·7
0·01 *M* glycerol	Nil	10·3	26·9
0·01 *M* glycerol	4	10·3	10·0
0·01 *M* Na propionate	Nil	11·7	10·0
0·01 *M* Na propionate	4	11·7	Nil
0·01 *M* Na acetate	Nil	10·5	Nil
0·01 *M* Na acetate	4	10·5	Nil

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acid is expressed in terms of μl. gas. It was estimated in the following manner. The fluid in the respiration flask at the end of the experiment was centrifuged. To 2 ml. of the supernatant fluid was added 1 ml. dinitrophenylhydrazine reagent (0·7 g. 2:4-dinitrophenylhydrazine hydrochloride dissolved in 250 ml. *N* HCl). This mixture was allowed to stand for 10 min. at room temperature and then 2 ml. *N* NaOH were added to it. After allowing it to stand further for a few minutes with a little shaking, 5 ml. water were added. The solution was compared colorimetrically with solutions prepared in a similar way from known quantities of pyruvic acid (see Jowett & Quastel [1937]). The Spekker photoelectric absorptiometer was used for the colorimetric comparisons.

It will be observed from the results quoted in Table 6 that pyruvic acid accumulates during the oxidation of propionic acid by the vitamin-deficient bacteria in the absence of added vitamin B_1, as it does in the case of lactic acid and glucose. It is probable, therefore, that the stimulating action of vitamin B_1 on propionic acid oxidation, noted in Table 4, is partially if not wholly due to the effect of this vitamin on pyruvic acid oxidation. Yet in the case of the oxidation of acetic acid, in which the stimulating action of the vitamin is much more pronounced than in the case of propionic acid, there is no evidence at all of pyruvic acid accumulation in the absence of the vitamin (Table 6).

Experiments were carried out using semicarbazide as a fixative. Pyruvic acid accumulation may be shown to take place in the presence of semicarbazide when the sodium salts of lactic, succinic and propionic acids are oxidized by the vitamin B_1-deficient bacteria. None accumulates, however, when sodium acetate is oxidized under the same conditions. Typical results are shown in Table 7. The absence of any accumulation of a ketonic acid when the bacterial

Table 7

Conditions as in Table 4; *M*/18 semicarbazide hydrochloride present; *p*H = 7·4; no vitamin B_1 added; 10·5 mg. dry wt. of bacteria.

Substrate present	Pyruvic acid liberated in 1 hr. μl.
0·01 *M* Na lactate	20·0
0·01 *M* Na succinate	20·2
0·01 *M* Na propionate	16·8
0·01 *M* Na acetate	Nil

oxidation of acetic acid takes place in the presence of semicarbazide and in the absence of the vitamin throws doubt on the view that the stimulating action of the vitamin on acetic acid oxidation is connected with the oxidation of pyruvic acid as an intermediate. The following evidence may also be considered.

(1) Examination of the results quoted in Table 3 shows that the ratio of O_2 consumption to acetic acid utilization is approximately 2, even in the absence of added vitamin B_1. If acetic acid were oxidized incompletely in the absence of added vitamin a ratio less than 2 would be expected. Thus, if it were oxidized to succinic acid the ratio would be 0·25, if to malic acid the ratio would be 0·5, and if to pyruvic acid the ratio would be 0·75. Since the experimental ratio is 2·0 the conclusion would be that acetic acid is completely oxidized in the absence of added vitamin B_1, but at a much smaller rate. Since traces of the vitamin must still remain in the bacteria the simplest conclusion accounting for the facts would be that vitamin B_1 is essential for the oxidation of the acetic acid molecule itself. It cannot be concluded from these experiments that pyruvic acid is not an intermediate in the oxidation of acetic acid, for it may be argued that in the

absence of added vitamin the oxidation of acetic acid is so slow that pyruvic acid is formed at a sufficiently feeble rate for it to be fully oxidized with the aid of the traces of vitamin B_1 still present in the bacteria. Admittedly, however, the absence of any accumulation of ketonic acid in the presence of a fixative such as semicarbazide throws doubt on the validity of this argument.

(2) The suggestion may be made that, in the absence of vitamin B_1, traces of intermediates accumulate during the oxidation of acetic acid by propionic acid bacteria and that these intermediates retard the oxidation of the fatty acid. Such intermediates may be succinic acid, *l*-malic acid and pyruvic acid. Experiment shows that the addition of small quantities of these substances to acetic acid has no retarding effect whatsoever on acetic acid oxidation. With succinic and *l*-malic acids an accelerating effect on the oxidation of acetic acid is observed. This phenomenon will be discussed in a later paper. With pyruvic acid no inhibition of acetic acid oxidation occurs, as shown by a typical result given in Table 8, in which pyruvic acid was still present at the end of the respiration

Table 8

Conditions as in Table 1

Substrates present	% acetate utilized in 1 hr.
0·01 *M* Na acetate	10·9
0·01 *M* Na acetate + 5 μg. cocarboxylase	41·5
0·01 *M* Na acetate + 0·0017 *M* pyruvate + 5 μg. cocarboxylase	46·0

period when the acetic acid analyses were made. There was thus ample ketonic acid present throughout the experiment to bring about the alleged retardation of acetic acid oxidation.

(3) If acetic acid oxidation by propionic acid bacteria takes place via the intermediate production of pyruvic acid, it follows from the facts (*a*) that acetic acid is completely oxidized, (*b*) that no pyruvic acid accumulates, that the rate of removal of pyruvic acid by the bacteria should be greater than or at least equal to the rate of utilization of acetic acid under the same experimental conditions. Experiments designed to test this conclusion show that pyruvate is utilized by the bacteria at about the same rate as acetic acid. These results are shown in Table 9. Whilst this result is not inconsistent with the view that

Table 9

Conditions as in Table 4

Substrates added	Vitamin B_1 added μg.	Wt. of bacteria mg.	Substrate removed in 1 hr. μl. gas
0·01 *M* Na acetate	4	13·4	428
0·01 *M* Na pyruvate	4	13·4	504
0·0033 *M* Na acetate	4	10·5	178
0·0033 *M* Na pyruvate	4	10·5	181

pyruvic acid may be formed during acetic acid oxidation, it follows (on this view) that pyruvic acid must be produced from acetic acid at an appreciable rate. It is, therefore, very surprising that none should appear in the absence of vitamin B_1.

(4) If a substrate is oxidized via pyruvic acid, it follows that during the oxidation of the substrate the enzyme dealing with pyruvic acid becomes either partially or wholly saturated with the ketonic acid. This will result in a lowering of the rate of utilization of any pyruvic acid initially added with the substrate, there being in effect less free enzyme available to cope with the added pyruvic

acid. Thus if a mixture of pyruvic acid and a substrate be presented to a bacterial suspension the rate of disappearance of the pyruvic acid will depend on the extent to which the pyruvic enzyme is saturated by the ketonic acid arising from oxidation of the substrate itself.

Experiments, of which typical results are given in Table 10, show that when pyruvic acid (0·0033 *M*) is mixed with lactic acid, succinic acid, fumaric acid or glycerol, vitamin B_1 being present throughout, there is almost complete inhibition of utilization by propionic acid bacteria of the pyruvic acid added initially. These highly inhibitory effects are quite consistent with the fact that these substrates give rise to small but demonstrable quantities of pyruvic acid during their oxidation (Tables 6, 7). They show that pyruvic acid is formed from them at a sufficient rate to saturate the enzyme involved. The admixture of propionic acid with pyruvic acid results in a definite inhibition of the rate of utilization of the latter, as would have been anticipated. The addition, however, of acetic acid to pyruvic acid results in no inhibition within the experimental error of these experiments. This result is opposed to the view that acetic acid is oxidized via pyruvic acid for the following reasons.

(*a*) Acetic acid, as already stated, is oxidized fast enough to produce pyruvic acid at a rate sufficiently high to saturate, partially or wholly, the pyruvic acid enzyme. Propionic acid, which is oxidized at a rate less than that of acetic acid, brings about a definite inhibition of the utilization of added pyruvic acid. The lack of inhibition due to acetic acid indicates that pyruvic acid is being produced from it either extremely feebly or not at all.

(*b*) If acetic acid is oxidized via pyruvic acid, succinic acid is presumably the first product of oxidation of the fatty acid. Succinic acid, however, inhibits the utilization of added pyruvic acid (Table 10). It follows, from the lack of inhibition due to acetic acid, that the rate of formation from it of succinic acid must be small. Considering the relatively rapid rate of oxidation of acetic acid, it seems unlikely, therefore, that it is oxidized to an appreciable extent via succinic and pyruvic acids.

Table 10

Conditions as in Table 1. 4 μg. vitamin B_1 present throughout.

Substrates added	$-Q_{pyruvate}$ (μl. pyruvate utilized per hr. per mg. dry weight bacteria)
0·0033 *M* Na pyruvate	14·5
+0·01 *M* Na *dl*-lactate	0·0
+0·01 *M* Na succinate	2·3
+0·0066 *M* Na succinate	3·9
+0·0033 *M* Na succinate	10·0
+0·01 *M* Na fumarate	0·0
+0·0066 *M* Na fumarate	0·0
+0·0033 *M* Na fumarate	1·4
+0·08 *M* Na malonate	15·1
+0·01 *M* Na formate	17·3
+0·01 *M* Na acetate	14·7
+0·01 *M* Na propionate	11·5
+0·01 *M* Na butyrate	15·4
+0·01 *M* Na α-glycerophosphate	14·1
+0·01 *M* glycerol	0·9

It will be noted from Table 10 that formic acid and butyric acid do not inhibit the utilization of pyruvic acid, nor is the latter affected by the addition of malonic acid or α-glycerophosphoric acid.

The conclusions to be drawn from these experiments are:

(1) Vitamin B_1 is necessary for the oxidation of acetic acid by propionic acid bacteria.

(2) There is as yet insufficient evidence to show that acetic acid is oxidized via pyruvic acid in presence of propionic acid bacteria, the available evidence being against this conclusion.

Oxidation of substrates other than fatty acids by propionic acid bacteria

As may be anticipated, those substrates the oxidations of which are known to proceed through pyruvic acid as an intermediate are oxidized by vitamin B_1-deficient propionic acid bacteria at rates[1] which are increased by the addition of vitamin B_1 to the bacterial suspensions. Typical results are shown in Table 11.

Table 11

Conditions as in Table 1

	Q_{O_2}	
Substrates added	In absence of added vitamin B_1	In presence of 4 μg. vitamin B_1
Nil	13·1	16·6
0·01 *M* Na pyruvate	20·5	38·8
0·01 *M* Na *dl*-lactate	35·6	52·8
0·01 *M* Na succinate	29·9	50·2
0·01 *M* Na fumarate	28·3	44·6
0·01 *M* Na *l*-malate	18·9	26·1
0·01 *M* glucose	23·5	34·3
0·01 *M* fructose	28·4	32·2
0·01 *M* Na *l*-glutamate	18·9	25·9
0·01 *M* Na α-ketoglutarate	16·5	18·1
0·01 *M* *dl*-alanine	16·6	20·6
0·01 *M* glycerol	28·8	35·7
0·01 *M* Na α-glycerophosphate	16·9	18·8
0·01 *M* Na hexosediphosphate	21·0	21·0
0·01 *M* Na citrate	15·8	16·8
0·01 *M* Na β-hydroxybutyrate	18·4	22·7

The oxidation of lactic acid by vitamin B_1-deficient propionic acid bacteria is stimulated by the addition of vitamin B_1 to an extent comparable with that occurring with the oxidation of pyruvic acid. Similar large accelerating effects due to the vitamin are to be noted with the oxidation of succinic and fumaric acids. It is, however, remarkable that the rate of oxidation of *l*-malic acid is less than that of fumaric acid and that the effect of vitamin B_1 addition is not nearly so pronounced as with succinic and fumaric acids. This result throws doubt on the generally accepted view that fumaric acid oxidation necessarily proceeds through the intermediate formation of malic acid. The fact that the rate of oxidation of fumaric acid may exceed that of malic acid has been noted before [Quastel & Wheatley, 1931] with *M. lysodeikticus* and *Ps. pyocyanea*.

This result, however, cannot be held as proof that fumaric acid oxidation, in the presence of propionic acid bacteria, proceeds independently of the formation of malic acid, as it is possible that fumarate may act catalytically in this oxidation. Thus, the following reactions may occur.

1. Fumaric acid $\rightleftarrows$ *l*-malic acid.
2. *l*-Malic acid + fumaric acid $\rightleftarrows$ oxaloacetic acid + succinic acid.
3. Succinic acid $\xrightarrow{O}$ fumaric acid.
4. Oxaloacetic acid $\rightarrow$ pyruvic acid + CO_2.

[1] I.e. the rates of oxygen uptake by the bacteria in presence of the substrates.

If reaction 2 is sufficiently rapid, a catalytic action of fumaric acid on malic acid oxidation will occur, its extent being dependent on the initial concentration of fumaric acid present.

A noteworthy fact is that α-ketoglutaric acid undergoes but little oxidation by propionic acid bacteria, its rate being scarcely influenced by the addition of vitamin B_1. Glutamic acid is oxidized at a somewhat faster rate and there is a definite, though feeble, stimulating action of vitamin B_1 on its oxidation. Alanine is but poorly oxidized, and vitamin B_1 has little effect on its rate of oxidation.

Both glucose and fructose are attacked by the vitamin B_1-deficient bacteria, but the addition of the vitamin has a much larger effect on the oxidation of glucose than upon that of fructose under the given experimental conditions.

It is interesting to note (Table 10) that whereas sodium α-glycerophosphate suffers little oxidative change by the bacteria, either in the presence or absence of vitamin B_1, glycerol is strongly attacked, the addition of vitamin B_1 having a marked effect on the rate of O_2 uptake. Sodium hexosediphosphate is oxidized at a rate which is appreciably faster than that of sodium α-glycerophosphate, but the rate is quite unaffected by the addition of vitamin B_1.

Sodium citrate and sodium β-hydroxybutyrate are oxidized but feebly, the addition of vitamin B_1 having little effect on the rates of O_2 uptake in presence of these substances.

Oxidation of ethyl and propyl alcohols by propionic acid bacteria

On testing methyl, ethyl and propyl alcohols as substrates for oxidation by vitamin B_1-deficient propionic acid bacteria, it was found that whereas methyl alcohol is not (or very feebly) attacked, ethyl and propyl alcohols are strongly oxidized. The addition of vitamin B_1 greatly increases the rate of oxidation of ethyl alcohol, but its effect on that of propyl alcohol is very much smaller. Typical results are shown in Table 12. The oxidation of ethyl alcohol leads to the

Table 12

Conditions as in Table 4

	Q_{O_2}	
Substrate	In absence of added vitamin B_1	In presence of 4 μg. vitamin B_1
Nil	10·5	12·4
0·033 *M* methyl alcohol	11·5	13·9
0·033 *M* ethyl alcohol	32·2	44·1
0·033 *M* propyl alcohol	61·7	66·2

Table 13

Conditions as in Table 1

Substrate	μl. acetic acid in 1 hr. in absence of added vitamin B_1	μl. acetic acid in presence of 4 μg. vitamin B_1
0·03 *M* ethyl alcohol	201·6	47·0

appearance of a volatile acid, presumably acetic acid. Estimations show that the formation of this acid is greatly reduced when the oxidation of ethyl alcohol by the bacteria takes place in presence of vitamin B_1 (Table 13). Doubtless the stimulant action of vitamin B_1 on ethyl alcohol oxidation is partly due to its powerful effects on the oxidation of acetic acid formed as intermediary.

Oxidation by propionic acid bacteria of substrates nearly related to acetic acid

It was of interest to examine the respiration of propionic acid bacteria in presence of substrates nearly related to acetic acid. Glycollic acid is oxidized at a low rate which is slightly increased by the addition of vitamin B_1. Glycine is oxidized at a higher rate with a small but definite accelerating action due to the addition of vitamin B_1. Neither of these substances is oxidized at a rate comparable with that of acetic acid in presence of the vitamin. Oxalic acid is oxidized either very feebly or not at all. These results are noted in Table 14.

Table 14

Conditions as in Table 4

Substrate added	Q_{O_2} In absence of added vitamin	Q_{O_2} In presence of 4 μg. vitamin B_1
Nil	10·5	12·4
0·01 *M* glycine	22·4	29·5
0·01 *M* Na glycollate	15·0	19·0
0·01 *M* Na oxalate	11·2	13·7
0·01 *M* Na acetate	23·4	65·3

Vitamin B_1 and the oxidation of α-ketobutyric acid

The addition of sodium α-ketobutyrate to vitamin B_1-deficient bacteria results in a rate of O_2 uptake approximately the same as that which occurs in the presence of sodium pyruvate. The addition of vitamin B_1, however, under the given experimental conditions results in little or no stimulation of the respiratory rate, in contrast to that which occurs with sodium pyruvate (see Table 15). Long & Peters [1939] found that with washed avitaminous pigeon brain the respiratory rate in presence of sodium α-ketobutyrate is stimulated to a small extent by the addition of vitamin B_1. Under different experimental conditions, which will be described in a later paper, we have found that there is a definite effect of vitamin B_1 on the oxidation of sodium α-ketobutyrate by the propionic acid bacteria. The effect, however, is much smaller than that occurring with pyruvic or acetic acid.

Table 15

Conditions as in Table 1

Substrate added	Q_{O_2} In absence of added vitamin B_1	Q_{O_2} In presence of 4 μg. vitamin B_1
Nil	13·1	16·6
0·01 *M* Na pyruvate	20·5	38·8
0·01 *M* Na α-ketobutyrate	19·0	21·7

Vitamin B_1 and cocarboxylase

Cocarboxylase is approximately as effective as vitamin B_1 in stimulating the rate of O_2 uptake by vitamin B_1-deficient bacteria in presence of sodium acetate. A characteristic feature of its activity, however, as is shown by typical results given in Table 16, is that its effects are a little less marked than those of vitamin B_1 at small concentrations and a little more marked at high concentrations. The fact

Table 16

Conditions as in Table 4. Substrate: 0·01 M Na acetate

	Q_{O_2}			
	0·3 μg.	1 μg.	5 μg.	10 μg.
Vitamin B_1	48·0	50·5	50·5	52·2
Cocarboxylase	37·6	41·8	53·4	56·2

that cocarboxylase activity exceeds that of vitamin B_1 at relatively high concentrations is evidence in favour of the conclusion that cocarboxylase, rather than vitamin B_1, is the actual catalytic agent. Silverman & Werkman [1939, 1, 4] have shown that propionic acid bacteria synthesize cocarboxylase from vitamin B_1. The fact that the activity of cocarboxylase in stimulating the respiration of propionic acid bacteria in presence of sodium acetate is feebler than that of vitamin B_1 at small concentrations may be attributed to a smaller rate of penetration of the pyrophosphoric acid ester into the bacterial cell.

Lipmann [1937] demonstrated the importance of cocarboxylase in the respiration of alkali-washed cells of *Bact. Delbruckii* in presence of sodium pyruvate. Barron & Lyman [1939] have found that cocarboxylase, rather than vitamin B_1, is the effective catalytic agent in the respiration of *Streptococcus haemolyticus* and of *M. gonorrhoeae* in a pyruvate medium.

Effects of components of vitamin B_1 on acetate oxidation by vitamin B_1-deficient propionic acid bacteria

It has been shown by Knight [1937, 1] that a mixture of 4-amino-5-aminomethyl-2-methylpyrimidine and 4-methyl-5-β-hydroxyethylthiazole is almost as effective as vitamin B_1 itself on the growth of staphylococci. Hills [1938] found that a mixture of the two components secures a large increase of O_2 taken up by vitamin B_1-deficient staphylococci in presence of sodium pyruvate. The components separately have no significant effect in raising the O_2 uptake.

A similar effect is found of the mixture of the two components on the oxidation of sodium acetate by vitamin B_1-deficient propionic acid bacteria. Typical results are shown in Table 17.

Table 17

Conditions as in Table 1. Substrate = 0·01 M Na acetate

Additions to the medium in the respiration flask	μl. O_2 uptake in 1 hr.
Exp. 1	
Nil	211·9
100 μg. pyrimidine component	215·6
100 μg. thiazole component	226·3
100 μg. pyrimidine component + 100 μg. thiazole component	435·0

Conditions as in Table 4. 0·01 M acetate throughout

Exp. 2	Q_{O_2}
Nil	28·8
0·2 μg. pyrimidine component	39·8
0·2 μg. thiazole component	27·5
0·2 μg. pyrimidine component + 0·2γ thiazole component	60·5
0·2 μg. vitamin B_1	62·3

These results make it clear that propionic acid bacteria are able to synthesize vitamin B_1 from a mixture of the two components, even when these are present

in low concentrations. Moreover, the results demonstrate that the components of vitamin B_1 are unable, separately, to exert catalytic effects on the oxidation of acetic acid by propionic acid bacteria. We have, however, found that under certain conditions the pyrimidine component can itself bring about an apparent stimulation of acetic acid oxidation (see, for example, exp. 2, Table 17). This finding is being further investigated. The phenomenon is probably similar to that described by Ochoa & Peters [1938] and explained by Westenbrink & v. Dorp [1940]. Silverman & Werkman [1939, 3] were unable to show a synthesis of vitamin B_1 from a mixture of the pyrimidine and thiazole components by propionic acid bacteria. Their pyrimidine component, however, differed from the one used in this work in having a $—CH_2Br$ group attached to the 5C atom of the pyrimidine ring instead of $—CH_2NH_2$. Conceivably, as Silverman & Werkman point out, linkage may not take place when the former group is attached to the pyrimidine ring.

Summary

1. The presence of vitamin B_1 at low concentrations (e.g. *ca.* $10^{-7}M$) greatly increases the rate of oxidation of acetic acid by vitamin B_1-deficient propionic acid bacteria. The ratio of mol. oxygen consumed to mol. acetic acid utilized by these bacteria, in the presence and in the absence of vitamin B_1, is approximately 2·0, showing complete oxidation of acetic acid.

2. Formic, acetic, propionic and butyric acids are oxidized at about the same rate by vitamin B_1-deficient propionic acid bacteria. The addition of vitamin B_1 has little or no effect on the oxidation of formic and butyric acid, but has a definite stimulating action on that of propionic acid. This effect is much less than that observed with the oxidation of acetic acid.

3. Pyruvic acid accumulates during the oxidation of glucose, lactic acid, glycerol and propionic acid by vitamin B_1-deficient propionic acid bacteria. Its amount greatly diminishes in presence of the vitamin. No pyruvic acid accumulates when acetic acid is oxidized.

4. In presence of semicarbazide a ketonic acid (probably pyruvic acid) accumulates during the oxidation of lactic, succinic and propionic acids. It does not accumulate during the oxidation of acetic acid.

5. The rate of utilization of acetic acid by propionic acid bacteria in presence of vitamin B_1 is approximately equal to that of pyruvic acid.

6. The presence of lactic acid, succinic acid, fumaric acid or glycerol greatly inhibits the utilization of pyruvic acid, when this is added together with any of these substrates, by propionic acid bacteria in presence of vitamin B_1. Propionic acid also produces a definite inhibition of added pyruvic acid utilization, but acetic acid gives no inhibition within limits of experimental error.

7. The presence of vitamin B_1 greatly stimulates the rates of respiration by vitamin B_1-deficient propionic acid bacteria with the following substrates: pyruvic acid, lactic acid, succinic acid, fumaric acid, glucose, glycerol. Definite but much less powerful effects of the vitamin are shown in the oxidations of *l*-malic acid, *l*-glutamic acid, *dl*-alanine, glycollic acid, glycine and fructose. The following substances are oxidized either feebly or not at all by the bacteria, with little or no stimulating action of vitamin B_1: oxalic acid, citric acid, β-hydroxybutyric acid. Oxidation of α-glycerophosphoric acid and hexosediphosphoric acid takes place with little or no effect of added vitamin B_1 on their rates of oxidation. Oxidation of fumaric acid takes place at a greater rate than that of *l*-malic acid.

8. The stimulant action of vitamin B_1 may, in most cases, be attributed partially if not wholly to its effect on the oxidation of pyruvic acid formed as an

intermediate. The effect of the vitamin on acetic acid oxidation cannot at present be ascribed to this cause.

9. α-Ketobutyric acid is oxidized slowly by vitamin B_1-deficient propionic acid bacteria, there being little acceleration on addition of the vitamin.

10. Ethyl alcohol is oxidized by vitamin B_1-deficient propionic acid bacteria, the rate of oxidation in its presence being greatly stimulated by the addition of vitamin B_1. Acetic acid accumulates during the oxidation of the alcohol, its amount being greatly reduced in presence of vitamin B_1. Probably the accelerating action of the vitamin on ethyl alcohol oxidation is due to its powerful effects on the oxidation of acetic acid formed as intermediary. Propyl alcohol is vigorously oxidized by vitamin B_1-deficient bacteria, but there is relatively little accelerating effect due to the addition of vitamin B_1. Methyl alcohol suffers little oxidative change in the presence or absence of vitamin B_1.

11. The evidence is in favour of the conclusion that cocarboxylase is the effective catalytic agent.

12. The pyrimidine and thiazole components of vitamin B_1 are, separately, unable to catalyse the oxidation of acetic acid by propionic acid bacteria. Together they are as effective as vitamin B_1 or cocarboxylase. Thus linkage of the two components will take place in presence of propionic acid bacteria.

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